New insights into immune mechanisms underlying response to Rituximab in patients with membranous nephropathy: A prospective study and a review of the literature

New insights into immune mechanisms underlying response to Rituximab in patients with membranous nephropathy: A prospective study and a review of the literature.

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Abstract

Background

Idiopathic membranous nephropathy (MN) is a common immune-mediated glomerular disease and the main cause of nephrotic syndrome (NS) in Caucasian adults. Rituximab (RTX) has been reported to safely reduce proteinuria in patients with primary MN and severe NS. However, the effects of RTX treatment on T–cells including regulatory T-cells (Treg) in MN have not been fully determined.

Methods

Seventeen patients [mean age 67 (29–86) years, 6 women, 11 men] with biopsy-proven MN, and persistent proteinuria > 3.5 g/24 h were prospectively enrolled and received RTX, 375 mg/m2 (iv) on days 1, 8, 15 and 22. Changes in circulating B and T cell homeostasis were examined in the peripheral blood by flow-cytometry studies; serum levels of IL-35 were measured using a high-sensitivity ELISA kits (baseline, at month 3, 6, 9 and 12).

Results

Patients had been followed-up for a mean of 36.3 months (24–48). Proteinuria decreased from 5.6 (3.5–8) g/24 h to 2.4 (0.06–13) g/24 h at 6 months ($p < 0.05$) and to 1.3 (0.06–8) at 12 months ($p < 0.01$), respectively after therapy with RTX. Four patients received a 2nd course of RTX (one at 6 months because of persistent NS, and three at 12, 18, or 30 months for relapse). The three relapsing patients became proteinuria-free (< 0.5 g/24 h) in the following 6 months. Serum creatinine remained stable during the follow-up: median 1 mg/dl (0.7–1.6) at 12 months and 1.1 (0.7–1.7) at 24 months as compared to 1 (0.5–2.4) at baseline. At 6 months after RTX, complete remission (CR) was observed in 7 patients, partial remission (PR) in 4, while 6 were non responders (NR) non responder (NR). At the end of the follow-up, 14 patients were in CR, 1 in PR, while 2 were NR. In the T-cell compartment, upon detection of B cell depletion, there was an increase in Treg up to 10-fold when comparing baseline and at month 12 (mean ± SD 1.2 ± 0.6%, and 5.8 ± 0.7% $p = 0.02$, respectively). When stratifying patients in responders (CR + PR) and NRs at month 12, we observed a significant increase in Treg cells from month 6 which persisted till 12 months only in the responder group (5.5 ± 0.6% and 1.1 ± 0.6%, $p = 0.04$, respectively in responders and NRs). A statistically significant decrease in the levels of active T-lymphocytes (HLA-DR + CD8 + cells) was observed, with a maximum reached at 12 months after treatment with RTX [6 ± 1.1% baseline, 4.7 ± 1.7% at 6 months ($p = 0.043$) and 1.5 ± 1.4% at 12 months ($p = 0.05$)]. A marked increase in IL-35 levels [defined as delta > 40% (serum values at 6 months minus baseline values)] was seen in 68% of the patients who achieved clinical response (CR or PR) at 12 month, but in none of the patients who failed to respond ($p = 0.034$).

Conclusion

Our findings and data from literature support the idea that RTX can be envisaged as a first-line therapy for patients at risk of progression because of persistent NS due to idiopathic MN. Insights into the putative T cell-related mechanisms of action have been discussed.

1. Introduction

Idiopathic membranous nephropathy (MN) is a common immune-mediated glomerular disease and remains the main cause of nephrotic syndrome (NS) in Caucasian adults [1], [2], [3], [4] and [5]. Although in most patients the disease progresses relatively slowly, about 40% of patients eventually develop end-stage renal disease (ESRD) [6] and [7]. Available immunosuppressive therapies include the use of corticosteroids combined with cytotoxic agents, and calcineurin inhibitors. These therapies are at least partially successful in reducing proteinuria, but their use is controversial, is associated with significant adverse effects and carries a high rate of relapse (reviewed in [3], [8] and [9]). These are important considerations in a disease where up to 30% of MN patients may achieve spontaneous remission of proteinuria with long-term renal survival with only supportive therapy [10]. Major advances in understanding the pathophysiology of MN have occurred since the early 2000s with the identification of neutral endopeptidase as the first human podocyte antigen involved in a rare subset of patients with neonatal alloimmune MN [11] and [12], followed by the characterization of phospholipase A2 receptor (PLA2R), another podocyte antigen targeted by circulating antibodies in 70%–80% of adult patients with primary MN. This major breakthrough showed that primary MN is an autoimmune disease in which the podocyte is the target and the source of the autoantigen [13]. Starting from these assumptions, in 2002, rituximab (RTX), a monoclonal antibody against the cell surface antigen CD20 of B cells [14], [15], [16], [17], [18], [19] and [20], was reported to safely reduce proteinuria and ameliorate NS in 8 patients with primary MN and severe NS unresponsive to prolonged angiotensin-converting-enzyme (ACE) inhibitor therapy [21]. Subsequent studies consistently confirmed these preliminary findings [22] even when RTX was

administered as a second-line treatment in patients who had previously failed to respond to steroids, alkylating agents, or calcineurin inhibitors or who had relapsed after transient remission [23] and [24]. Finding that RTX therapy achieved disease remission and stabilized or even improved renal function in 100 patients at high risk of poor outcomes because of persistent NS pointed to a pathogenic role of antibody-producing lymphocytes in primary MN [25]. Indeed, experimental and human data converge to indicate that deposition along the glomerular basement membrane of immunoglobulins produced by autoreactive B cells initiates the sequence of events resulting in secondary injury to the glomerular filtering barrier and proteinuria [26]. Therefore, agents that specifically interfere with B cell antibody production would ideally be the first step toward selective therapy for primary MN. Recently, successful treatment with RTX has been associated with regression of some T-cell abnormalities in patients with autoimmune conditions such as chronic ITP [27] and SLE [15]. Consistently, data from our group showed a T-lymphocytes re-assessment after RTX in patients with severe lupus nephritis [15]. However, the effects of RTX treatment on T–cells including regulatory T-cells (Treg) in MN have not been fully determined. These considerations prompted us to critically review the most recent literature about the role of T-lymphocytes in MN pathogenesis. Similarly, in order to further define immune mechanisms underlying response to B-cell depletion, we prospectively assessed lymphocytes re-assessment (including CD4 + FOXP3+Tregs) and IL-35 levels as predictors of response to RTX in patients with MN. Additionally, the observed clinical outcome results were compared to those emerging from the updated reviews of the literature on this topic.

2. Predictors of response in MN patients treated with RTX

Given the variability in the natural history of the disease, historically, an approach in MN has been to limit immunosuppression treatment to those subjects identified as being at higher risk of progression, and a number of predictors of renal outcome and disease progression have been identified for patients with idiopathic MN [28] and [29]. However, in treated patients, little is known regarding factors that may predict response to therapy, especially in regard to the use of RTX.

Few studies have evaluated the use of urinary markers for this purpose. Bazzi et al. [30] quantified urinary IgG and α1-microglobulin in 38 patients with NS and normal renal function. Using an arbitrary cutoff value, these authors showed that 100% of patients with a baseline IgG excretion of < 110 mg/g urinary creatinine (uCr) underwent remission of proteinuria versus 20% in those with an IgG excretion of > 110 mg/g uCr. Similarly, 77% of the patients with an α1-microglobulin of < 33.5 mg/g uCr went into remission versus 17% of the patients with an α1-microglobulin of > 33.5 mg/g uCr. Of note, the remission rate was independent of baseline proteinuria. Of the 38 patients, 19 were allocated, in a non-randomized fashion, to receive either corticosteroids and cyclophosphamide for 6 months (n = 16) or corticosteroids alone (n = 3), versus continuation of conservative therapy (n = 19). There was no difference in remission of proteinuria between the treated and the untreated patients. A greater percentage of patients with urinary IgG and α1-microglobulin above the cutoff value went into remission with immunosuppressive therapy versus those on conservative treatment, but the results did not reach statistical significance [30]. More recently, Fervenza et al. studied 20 patients with MN treated with RTX [31]. Of the 18 patients who completed a 24-month follow-up, remission of proteinuria was seen in 16 patients. However, as previously mentioned, baseline proteinuria, pharmacokinetic studies and evaluation of B and T cell subsets could not predict which patient would respond to RTX. In a subsequent study, the same groups showed that in patients with MN, quantification of low-, medium- and high-molecular-weight urinary proteins may be associated with rate of response to RTX. However, this quantification does not correlate with longer term outcomes [32]. The identification of anti-PLA2R antibodies posed the question whether these antibodies could a priori identify patients who may respond to RTX and thus benefit from this form of therapy. Several studies [33], [34], [35] and [36] indicate that in patients with MN treated with either cyclophosphamide or tacrolimus associated with RTX, the anti-PLA2R antibody titer dynamics after treatment initiation preceded NS remission and correlated with the treatment response. Very recently, these observations were confirmed by Ruggenenti and coworkers [37]. In a cohort of 132 RTX-treated patients (among those 81 had anti-PLA2R antibodies), lower anti-PLA2R antibody titer at baseline ($p = 0.001$) and full antibody depletion 6 months post-RTX (hazard ratio [HR], 7.90; 95% confidence interval [95% CI], 2.54 to 24.60; $p < 0.001$) strongly predicted remission. They concluded that assessing circulating anti-PLA2R autoantibodies and proteinuria may help in monitoring disease activity and guiding personalized RTX therapy in nephrotic patients with primary MN. The same study showed that the kinetics of circulating CD20+ cells or anti-PLA2R autoantibody depletion and proteinuria reduction after RTX therapy were similar in patients receiving the standard four 375 mg/m2 dose protocol or a B cell-driven regimen [38]. These data may be of clinical relevance for several reasons. First, one could consider that avoiding unnecessary re-exposure to RTX, in addition to be extremely cost-saving, might also limit the production of anti-chimeric antibodies that may increase the risk for adverse reactions and prevent retreatment of disease recurrences. Secondly, conversely to what observed in other autoimmune disease [39], Remuzzi and co-workers showed that sustained B-cell depletion seemed to be achievable in MN with a single infusion of RTX; additional research to confirm these observations is warranted [38].

3. Aim of the study

The present study aimed to prospectively evaluate whether in patients with MN early changes in lymphocytes profile and IL-35 level, the newest identified member of the interleukin-12 cytokine family, could predict response to RTX in patients with MN.

4. Materials and methods

4.1. Patients

Patients included in the study met the following criteria: (i) biopsy-proven MN; (ii) creatinine clearance (CrCl) ≥ 30 ml/min/1.73 m2 and (iii) persistent proteinuria > 3.5 g/24 h despite maximal tolerated angiotensin II blockade for at least 4 months. Patients with active infection, diabetes or a secondary cause of MN were excluded. Patients who had been on treatment with prednisone, cyclosporine or mycophenolate mofetil within the last 4 months, or alkylating agents within the last 6 months were also excluded from the study.

Seventeen patients (mean age 67 (29–86) years, 6 women, 11 men) who fulfilled the above inclusion criteria were prospectively enrolled and received RTX, 375 mg/m2 (iv) on days 1, 8, 15 and 22.

4.2. Follow-up

In all patients, clinical and laboratory parameters, including complete blood counts, electrolytes, serum albumin, serum IgG (IgM, IgA), and a lipid panel, were evaluated at study entry, and at months 3, 6, 9, 12, 18 and 24. Protein excretion in the urine was assessed by performing two consecutive 24 h at study entry, and at months 3, 6, 9, 12, 18 and 24.

Immunological analyses (including B-cell, T-cell, and regulatory T-cell analysis) and IL-35 dosage were performed as described below. Definition of remission status is according to the criteria established by Cattran et al. [40]. CR was defined as proteinuria ≤ 0.3 g/24 h, PR as sub-nephrotic (≤ 3.5 g/24 h) proteinuria with a > 50% reduction in peak proteinuria along with serum albumin > 3 g/dl, and nonresponse (NR) as > 50% reduction in peak proteinuria. Any case reaching a CR or PR was considered as a treatment success.

4.3. B and T cell subset analysis

Circulating B cells in the peripheral blood were investigated by CD20 + and CD19 + B cells analyzed by flow-cytometry at baseline, month 1, month 2 and every other month thereafter. Changes in T cell homeostasis following RTX-induced B cell depletion were examined including flow-cytometry studies at baseline (before the first RTX infusion), at month 3, 6, 9 and 12. Whole blood samples collected in EDTA in the morning were stained with monoclonal antibodies against CD45 (APC 100 eBioscience Bender Medsystems, CA, USA), CD3 (FITC eBioscience Bender Medsystems, CA, USA), CD4 (PC7 Beckman Coulter, CA, USA), CD19 (Pacific Blue™, Beckman Coulter, CA, USA), CD20 (PE Beckman Coulter, CA, USA), CD25 (PerCP-eFluor 710 eBioscience/Bender Medsystems, CA, USA), FOXP3 (PE Staining set, eBioscience Bender Medsystems, CA, USA), CD28 (PE Staining set, eBioscience Bender Medsystems, CA, USA), CD8 (PB Staining set, eBioscience Bender Medsystems, CA, USA), and HLA-DR (PERPC-710, eBioscience Bender Medsystems, CA, USA).

4.4. IL-35 and anti-PLA2R detection

Serum levels of IL-35 in MN patients were measured in duplicate using a high-sensitivity ELISA kit (Kit A, BioLegend MAX Human IL-35 heterodimer ELISA kit, San Diego, CA, US). Results were confirmed using a second ELISA kit (Human IL-35 heterodimer ELISA kit, Cloud-Clone Corp. Houston, TX, USA). There was a positive correlation between Kit A and Kit B by Spearman test (R2=0.7197, p=0.046) (Fig. 1).

nalysis was performed at baseline (before the first RTX infusion), 3, 6, 9, 12, and 18 months. The assay recognizes natural human IL-35 protein, and its limit of detection is 6 pg/ml. Serum levels of IL-35 were also measured in 20 healthy donors. Similarly, serum samples were tested at baseline (before the first RTX infusion), 3, 6, 9, 12, and 18 for the presence of anti-PLA2R. The immunofluorescence test uses biochips coated with HEK293 cells transfected with the full-length human PLA2R1 cDNA and incubated with a serum sample from the patient. Sample titration was used when appropriate (UROIMMUN AG, Luebeck, Germany).

5. Statistical analysis

For comparison of variables at baseline and follow-up, a Student's t-test was used for normally distributed parameters, and the non-parametric Mann–Whitney test for non-normally distributed parameters. Correlations were calculated and significance determined by Fisher's test. Multivariable logistic regression analysis was used to identify any independent predictors of response to RTX. For these analyses, the Prism (GraphPad Software, CA, USA) and SPSS (IBM Corporation, NY, USA) software programs were used. $p < 0.05$ was considered significant.

6. Results

6.1. Clinical outcome

In MN patients proteinuria decreased from 5.6 (3.5–8) g/24 h to 2.4 (0.06–13) g/24 h at 6 months ($p < 0.05$) and to 1.3 (0.06–8) at 12 months ($p < 0.01$), respectively after therapy with RTX. Four patients received a 2nd course of RTX (one at 6 months because of

persistent nephrotic syndrome, and three at 12, 18, or 30 months for relapse). The three relapsing patients became proteinuria-free (< 0.5 g/24 h) in the following 6 months. The unresponsive patient was lost from the follow-up.
Serum creatinine remained stable during the follow-up: median 1 mg/dl (0.7–1.6) at 12 months and 1.1 (0.7–1.7) at 24 months as compared to 1 (0.5–2.4) at baseline.
At 6 months after RTX, CR was observed in 7 patients, PR in 4 and NR in 6. The response rate at 12, 18 and 24 months are shown in Fig. 2. At the last visit, after a mean of 36.3 months (24–48), 14 patients were in CR, 1 in PR and NR 2. One unresponsive patient was lost from the follow-up.

6.2. Relationship of peripheral blood B cells and clinical response

Initial CD20 B-cell complete depletion was seen in all patients after RTX. Overall, CD20 B-cells recovered within 12 months [median time to re-population: 12 months (range 6–14)]. As previously reported [31], we observed that B-cell depletion in patients with MN receiving RTX was not as sustained as seen in patients with other conditions (e.g. ANCA associated vasculitis) treated with an identical dosing regimen, since at 6 months 4 out of 17 patients showed an initial B-cells repopulation (circulating CD20+ B-lymphocytes 1.6%–4.3%). In response to the second cycle, all 4 patients again showed complete depletion of peripheral B-cells. There was no significant correlation between baseline CD20 B-cell counts and change in proteinuria at 6, 12 or 24 months. Similarly, we did not observe any association between baseline CD20 B-cell and time to response in proteinuria, or the severity of initial proteinuria.

6.3. Analysis of T-cells subsets

A representative panel of quantification of B-cell, T-cell, and T subsets is presented in Fig. 3. In patients with MN, T-cell subset count was within the normal reference range for our laboratory at baseline. In the T-cell compartment, upon detection of B-cell depletion, there was an increase in Treg (CD4+ CD25+FOXP3+) up to 10-fold when comparing baseline and at month 12 (mean ± SD 1.2 ± 0.6%, and 5.8 ± 0.7% p=0.02, respectively). When stratifying patients in responders (CR + PR) and non-responders at month 12, we observed a significant increase in Treg cells from month 6 which persisted till 12 months only in the responder group (5.5 ± 0.6% and 1.1 ± 0.6%, p=0.04, respectively in responders and non-responders) (Fig. 4).
A statistically significant decrease in the levels of HLA-DR + CD8 + cells (active T lymphocytes) was observed, with a maximum reached at 12 months after treatment with RTX [(6 ± 1.1% baseline, 4.7 ± 1.7% at 6 months (p = 0.043) and 1.5 ± 1.4% at 12 months (p = 0.05)].
We failed to observe any difference when dividing patients for clinical response. However, in the two patients who failed to respond to RTX, the percentages of HLA-DR + CD8 + cells assessed at month 12 were higher when compared to the three faster responders, whose level of proteinuria was ≤ 0.3 g/24 h within the first 6 months after RTX (3.7% and 3.1% in the non-responders and 1.5%, 0.7% and 1.2% in the fast responders, respectively).

6.4. Relationship of IL-35 level, anti-PLA2R and clinical response

Overall, at 6 months after RTX serum levels of IL-35 were increased compared to baseline values [median 162.37 pg/ml (99.2–247.1) vs. 74.63 pg/ml (49.96–111.96), p = 0.04]. Besides, a more marked increase [defined as delta > 40% (serum values at 6 month minus baseline values)] was seen in 68% of the patients who achieved clinical response (CR or PR) at 12 month, but in none of the patients who failed to respond (p = 0.034). Moreover at month 6, all the three fast responders patients had IL-35 levels above the 75th percentile (219.6 pg/ml) of the distribution, while all the four patients who received a 2nd course of RTX had IL-35 below the median value (162.37 pg/ml).
When comparing serum levels of IL-35 at baseline, the patients with positive anti-PLA2R antibodies (69%, N = 9/13) showed lower IL-35 level than the negative anti-PLA2R patients (31%, N = 4/13) [median 129.63 pg/ml (99.2–165.5) vs 219.87 pg/ml [114.2–247.1] p = 0.031]. Of note, at 6 months after the last RTX infusion, a significant increase of IL-35 levels in the anti-PLA2R was observed only in the positive group [median 229.47 pg/ml [118.9–267.2] vs 189.67 pg/ml (102.2.–237.5), p = 0.029].
All these observations were consonant with the concept that IL-35 may influence the proliferation and function of effector T cells, since overall in our cohort, upon B cell depletion, the serum levels of IL-35 have been found to be increased compared to baseline values [41], [42] and [43].

7. Discussion

About 20% of cases of NS in Caucasian adults are represented by MN. It is the third leading cause of ESRD in patients with primary glomerulonephritis, and is also the glomerulopathy that recurs most frequently after kidney transplantation. About 70% of patients present with NS, and the remainder with asymptomatic subnephrotic proteinuria. Sixty percent of patients progress to full NS.
MN is a chronic disease that can include spontaneous remission and relapsing course. Spontaneous remissions occur in one third of cases, usually within the first 2 years after presentation [44]. The predictors of spontaneous remission are sub-nephrotic proteinuria, female sex, age younger than 50 years, and preserved renal function at presentation [45]. The other two-thirds of patients are equally distributed into those with persistent proteinuria, who maintain renal function in the long-term, and patients who progress to renal failure [46].
The specific glomerular lesion consists of variable thickening of the glomerular capillary walls on light microscopy as a result of immune complex formation in the subepithelial space. The disease is staged according to the extent to which the subepithelial

immune deposits are surrounded by the glomerular basement membrane. The immune deposits include IgGs, and the complement membrane attack complex C5b-9 that is the mediator of proteinuria. C5b-9 triggers production of reactive oxygen species, directly or via local lipid peroxidation, release of proteases, and cytoskeletal changes with podocyte detachment.
MN can be idiopathic (more than 80% of cases), or secondary to cancer, hepatitis B or C, systemic lupus erythematosus, and drugs [47] and [48]. Clinical outcome is unpredictable. Treatment consists of potentially toxic agents and remains challenging [49].
Idiopathic MN has been recognized as an autoimmune disease in which the podocyte is the source of the autoantigen. Antibodies directed at the phospholipase A2 receptor have been detected in 70% of adult patients with idiopathic membranous nephropathy [13]. Molecular mimicry of PLA2R epitopes by microbes or other environmental antigens could lead to production of anti-PLA2R antibodies in genetically predisposed patients who have HLA-DQA1 risk variants [50]. PLA2R acts as a clearance receptor with potentially anti-inflammatory activity, which might be affected by an anti-PLA2R antibody. On the other hand, PLA2R regulates cellular senescence through production of reactive oxygen species and activation of DNA-damage pathways [48]. The potential agonistic activity of anti-PLA2R should be also considered in the context of the overexpression of podocyte senescence in patients with membranous nephropathy [51].
Another putative autoantigen is thrombospondin type-1 domain-containing 7A (THSD7A), and circulating autoantibodies against this protein were detected in a subset of patients negative for anti-PLA2R [48]. Patients with idiopathic membranous nephropathy have an autoimmune response against either PLA2R or THSD7A, but not both.
Non-podocyte antigens are involved in secondary membranous nephropathy, including cationic bovine serum albumin (detected in children younger than 5 years as a consequence of early exposure to bovine products), hepatitis B and C viral antigens, and enzymes used in the replacement of the deficient enzyme in lysosomal storage diseases [52], [53] and [54].

Although C5b-9 membrane attack complex is a major mediator of proteinuria, the predominant T-helper-2 immune response leads to the production of IgG4, which does not activate complement [55] either by the classical or the alternative pathways. Mannan-binding lectin has been identified in glomeruli from idiopathic membranous nephropathy patients [56]. Under-galactosilated anti-PLA2R IgG4 [56] could bind mannan-binding lectin and activate the lectin pathway [8]. Reduced activity of circulating or podocyte-associated complement regulatory proteins and intrinsic or acquired defects of complement receptor-1, due to genetic polymorphisms, might further influence the histologic and the clinical expression of the disease.
The presence or absence of anti-PLA2R autoantibodies does not predict the response to immunosuppressive therapy in idiopathic membranous nephropathy, but immunological remission is a strong predictor of clinical remission in treated patients. Indeed, anti-PLA2R antibody levels are correlated with proteinuria, and antibodies usually become undetectable at remission and re-emerge at relapse [13], [33], [34], [37], [57], [58], [59] and [60]. Moreover, high titers correlate with a decreased possibility of remission [58] and [60], an increased risk of development of NS [61], renal failure [59] and a delayed response to immunosuppressive therapy [60]. Decrease or disappearance of anti-PLA2R antibodies precedes renal remission by months [60] and [62], while the rate of remission is inversely related to antibody titer [60] and occurrence of complete remission is preceded by complete antibody depletion. Re-emergence of antibodies precedes renal relapse [60], while relapse often occurs when patients have persisting circulating antibodies at the end of immunosuppressive treatment [63]. Thus anti-PLA2R antibody titers might have a role in the selection of patients for immunosuppressive therapy addressing toward a more personalized care which will be more and more based on therapies more specific and less toxic than conventional immunosuppressants.
Anti-CD20 antibody represents a first step in this direction. RTX can safely reduce proteinuria both in naive patients and in patients unsuccessfully treated with steroids, alkylating agents, or calcineurin inhibitors, or relapsing after transient remission [23] and [25]. Amelioration of clinical signs and histological changes are paralleled by decreased glomerular IgG4 and C3 staining, and reabsorption of electron-dense subepithelial deposits together with increase in electron-dense diaphragms (which correlates with decrease in albumin fractional clearance) [64].
Treatment protocols for MN patients vary widely among different centers. The main studies about the use of RTX in MN are summarized in Table 1. Some authors used four 375mg/m2 weekly doses (lymphoma protocol), others preferred administration of 1 g RTX 2 week apart (rheumatoid arthritis protocol) [65]. Serial measurements of circulating B cells show that in patients with MN, lupus, vasculitis or mixed cryoglobulinemia CD20 cells are fully depleted from the circulation after the first drug administration [66] and [67], but B cell depletion lasts very longer in the so called ‘extended protocol’ [15], [39], [68] and [69].
Despite the lack of data on direct comparison in the context of RCTs, several reports from different groups have shown that RTX achieves complete or partial remission in approximately 70% of patients with primary MN, an effect that is similar to that reported for combination therapy with steroid and alkylating agents [22] and superior to calcineurin inhibitors [70]. We showed in one of the longest follow-up study available that RTX achieved 80% remission (including 10% partial remission) at 18 months. Remission has been heralded in every case by an increase in interleukin 35, with a definite relationship between the higher values and the faster response, paralleled by an increase of Treg which persisted over time.

Beside the recognized involvement of auto-reactive, antibody-producer lymphocytes, a role for regulatory T-cells in disease onset, remission and relapse has been proposed [71]. Insights into these mechanisms will possibly open new therapeutic avenues. At present, the effects of RTX on Treg [72] and [73] could strengthen the B-cell depletion and sustain the clinical response.
Our findings in conjunction with data from literature on effectiveness and safety of RTX support the idea that RTX can be envisaged as a first-line therapy for patients at risk of progression because of persistent nephrotic syndrome due to idiopathic membranous nephropathy.

Take-home messages

• RTX can be envisaged as a first-line therapy for patients at risk of progression because of persistent NS due to idiopathic MN -T cell-related mechanisms may play a role in modulating the clinical response to RTX in MN

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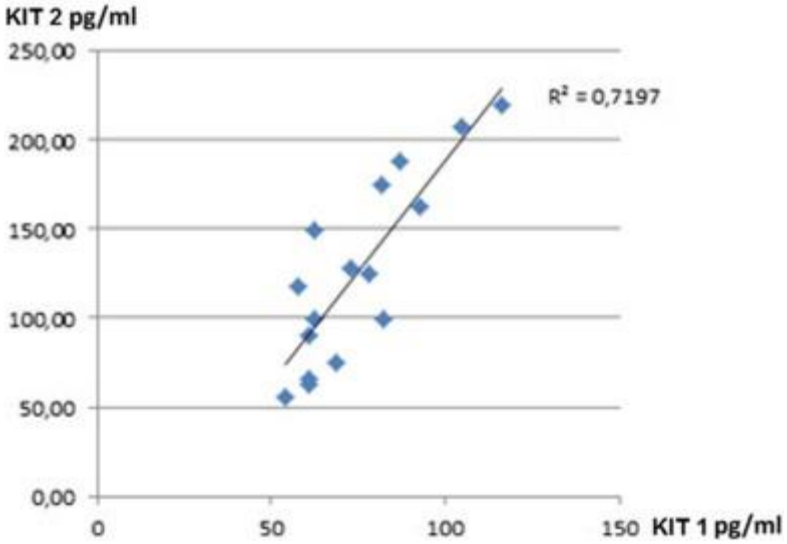

Fig. 1. Correlation analysis by Spearman test for Kit A and Kit B used for IL-35 detection.

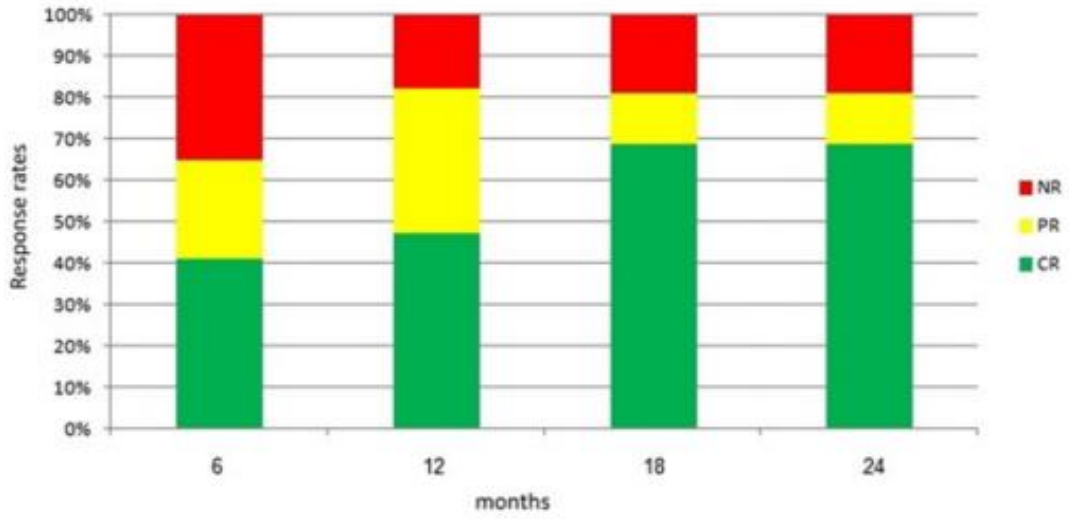

Fig. 2. Response rates (complete remission, CR; partial remission, PR; no response, NR) in patients with MN treated with RTX assed at 12, 18 and 24 months.

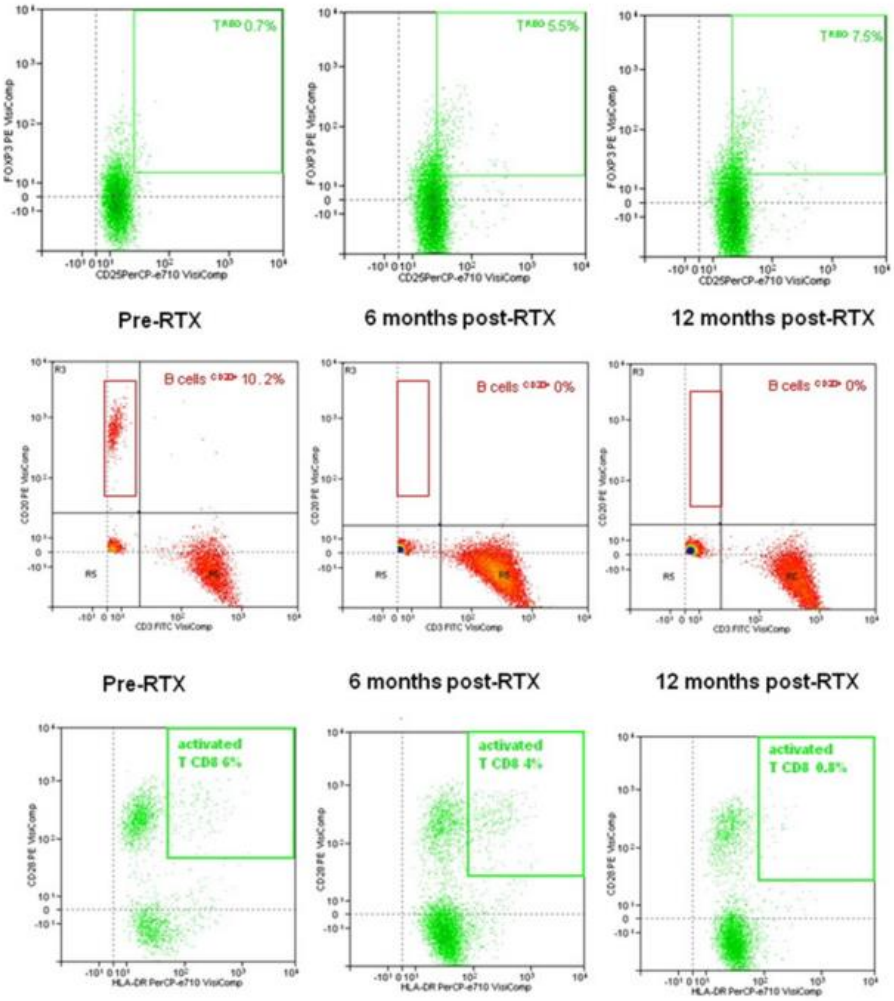

Fig. 3. Representative dot-plots of Treg (CD4 + CD25 + FOXP3 +, upper plots), active T-lymphocytes (HLA-DR + CD8 + cells, central plots), and B-cell (CD20 +, lower plots) as evaluated by flow-cytometry. Samples from a responder without relapse were analyzed before and 6, and 12 months after the B-cell depletion therapy.

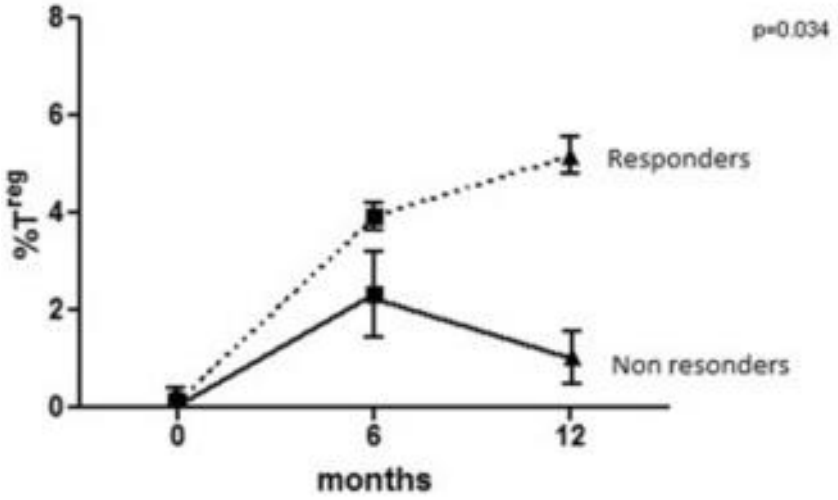

Fig. 4. Treg changes assessed at baseline, 6 and 12 months after RTX when stratifying patients in responders and non-responders to RTX.

Table 1 Study characteristics and clinical outcome of rituximab-treated patients with MN.

Name	Year	Study design	N	Anti-PLA_2R positivity	Associated drugs	Indications	RTX protocol	Proteinuria before Rituximab (mean g/24 h)	Renal function before RTX (mean)	Follow-up	Complete remission[a]	Partial remission[b]	Mean % reduction in proteinuria	AEs
Remuzzi et al.	2002	CS	8	N/A	ACE-I	Persistent NS	4 weekly (375 mg/m^2)	8.6	Cr 1.4 mg/dl	20 wk	2/8 (proteinuria ≤1 g/24 h)	3/8 (proteinuria >1 g/24 h and ≤3.5 g/24 h)	−57%	Generalized chills ($n = 1$), skin rash ($n = 1$), and questionable laryngospasm ($n = 1$)
Ruggenenti et al.	2003	PCS	8	N/A	ACE-I	Persistent (>6 mo) urinary protein excretion >3.5 g/24 h	4 weekly (375 mg/m^2)	8.6	Cr 1.4 mg/dl	1 yr	2/8 (proteinuria ≤1 g/24 h)	3/8 (proteinuria >1 g/24 h and ≤3.5 g/24 h)	−65%	Generalized chills ($n = 1$), skin rash ($n = 1$), and questionable laryngospasm ($n = 1$)
Ruggenenti et al.	2006	CR	23	N/A	ACE-I titrated to maximum dose, with ARB added after 3 mo	Persistent NS	4 weekly (375 mg/m^2)	9.1	Cr 1.4 mg/dl	1 yr	6/23 (proteinuria ≤1 g/24 h)	6/23 (proteinuria >1 g/24 h and ≤3.5 g/24 h)	−71%	N/A
Cravedi et al.	2007	CS	12	N/A	ACE-I titrated to maximum dose	Persistent NS	375 mg/m^2 1 ($n = 11$), 375 mg/m^2 2 ($n = 1$)	10.3	Cr 1.4 mg/dl	1 yr	2/12 (proteinuria ≤0.3 g/24 h)	6/12 (proteinuria >1 g/24 h and ≤3.5 g/24 h with a 50% reduction from baseline)	−60%	Nausea, chills, sweating, and face rush ($n = 1$)
Fervenza et al.	2008	CS	15	N/A	ACE-I and ARB	Persistent NS	1 g × 2, on days 1 and 15; repeated at 6 mo if proteinuria >3 g/24 h and CD19 B-cells >15/l ($n = 10$)	13	Cr 1.4 mg/dl	1 yr	2/15 (proteinuria ≤0.3 g/24 h)	6/15 (proteinuria >1 g/24 h and ≤3.5 g/24 h with a 50% reduction from baseline)	−48%	Itching, rigors, and skin rash ($n = 3$); sore throat ($n = 3$); muscle pain ($n = 1$);serum sickness-like syndrome ($n = 1$); hair loss/thinning ($n = 2$); community acquired pneumonia ($n = 1$); fatigue and voice loss ($n = 1$); reactivated herpes zoster ($n = 1$); and adenocarcinoma of lung with normal chest X-ray 1 yr before enrollment ($n = 1$)
Ruggenenti et al.	2008	CS	7	N/A	ACE-I titrated to maximum dose	Persistent NS	4 weekly (375 mg/m^2)	5.05	Cr 1.0 mg/dl	7–59 mo	7/7	0/7	−95%	N/A
Segarra Medrano et al.	2009	CS	13	N/A	ACE-I or ARB	Long-term CNI dependence	4 weekly (375 mg/m^2)	2.05	Cr 1.7 mg/dl	30 mo	13/13	0/13	−68%	N/A
El-Zoghby et al.	2009	CS	8	N/A	ACE-I and ARB	Persistent NS	1 g × 2, on days 1 and 15	2.11	Cr 1.6 mg/dl	2 yr	6/7	0/7	N/A	One patient had pneumonia 12 months following treatment and one patient developed histoplasmosis 9 months post-RTX
Fervenza el al.	2010	CS	18	N/A	ACE-I and ARB	Proteinuria >5 g/24 h	4 weekly (375 mg/m^2)	11.09	CrCl 72.4 ml/min	2 yr	4/18	12/18	−84%	The adverse events observed were mainly infusion-related reactions and none were serious (itchy throat, nasal congestion, and face flushing, flu-like symptoms minor skin rash) possible community-acquired pneumonia 2.5 months. One patient had a myocardial infarction 5 months after the first set of infusions
Beck et al.	2011	CS	35	71%	N/A	Proteinuria >5 g/24 h	1 g × 2, on days 1 and 15, repeated at 6 months if indicated. 4 weekly (375 mg/m^2) in 20 cases	11.5	Cr 1.5 mg/dl	2 yr	9/35	16/35	−65%	N/A
Cravedi et al.	2011	CC	22	N/A	N/A	Proteinuria >1 g/24 h, 11 with second line RTX, and 11 first line RTX	4 weekly (375 mg/m^2) in 5 cases; 6 patients received the B-cell-driven protocol.	10.6	Cr 1.2 mg/dl	2 yr	5/22	10/22	−58%	A transient facial rash during rituximab infusion (1)
Ruggenenti et al.	2012	PCS	100	N/A	ACE-I	Persistent NS	4 weekly (375 mg/m^2)	9.01	Cr 1.2 mg/dl	29 mo	27/100	38/100	N/A	No treatment-related serious adverse events occurred
Kong et al.	2012	RCS	11	N/A	ACE-I and ARB	Persistent NS	Single RTX (5 cases) infusion, 2 patients 2 infusions and 4 patients 4 infusions with cumulative doses of 500 mg	N/A	N/A	31.5 mo	7/11	0/11	N/A	Skin rash, throat irritation, chest tightness, difficulty in breathing, hypotension, bradycardia, and body ache
Bush et al.	2013	CC	14	N/A	N/A	Refractory NS	4 weekly (375 mg/m^2)	5.5	CrCl 53 ml/min	1 yr	3/14	7/7	−67%	N/A
Segarra Medrano et al.	2014	CC	53	Anti-PLA_2R antibody titer 420 RU/ml	Tacrolimus	Persistent NS	4 weekly (375 mg/m^2) 23 cases and 1 g × 2, on days 1 and 15, 30 cases	N/A	Cr 0.87 mg/dl	1 yr	28/53	21/53	N/A	N/A
Souqiyyeh et al.	2015	RCS	25	N/A	N/A	Persistent NS	4 weekly (375 mg/m^2)	N/A	N/A	18.0 ± 6.97	10/25	7/25	N/A	The rate for hospitalizations with serious infection was very low
Roccatello et al.	2015	PCS	17	69%	ACE-I and/or ARB	Persistent NS	4 weekly (375 mg/m^2)	5.6	Cr 1 mg/dl	36.3 mo	14/17	1/17	−77%	No major side effects. 2 infusion-related reactions (transitory skin rashes)

CS, case series; CC, case control; N/A, not available; RCS, retrospective cohort study; PCS, prospective case series; NS, nephrotic syndrome; ACE-I, angiotensin-converting-enzyme inhibitor; ARB, angiotensin receptor blockers; and CNI, calcineurin inhibitor; mo, months; yr, years; wk, week. a Complete remission: definition per study, otherwise proteinuria ≤0.3 g/24 h. b Partial remission: definition per study otherwise ≥50% reduction in proteinuria with proteinuria ≤3.5 g/24 h.